Use of RNase H and primer extension to analyze RNA splicing

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ABSTRACT

A new method for the characterization of pre-mRNA splicing products is presented. In this method RNA molecules are hybridized to an oligodeoxynucleotide complementary to exon sequences upstream of a given 5' splice site, and the RNA strands of the resulting RNA:DNA hybrids are cleaved by RNase H. The cleaved RNAs are then subjected to primer extension using a ³²P-labelled primer complementary to exon sequences downstream of an appropriate 3' splice site. Since the primer extension products all terminate at the site of RNase H cleavage, their lengths are indicative of the splice sites utilized. The method simplifies the study of the processing of complex pre-mRNAs by allowing the splicing events between any two exons to be analyzed. We have used this approach to characterize the RNAs generated by expression of the rat tropomyosin 1 (Tm 1) gene in various rat tissues and in cultured cells after transient transfection. The results demonstrate that this method is suitable for the analysis of alternative RNA processing *in vivo*.

INTRODUCTION

RNA splicing is commonly analyzed by S1 nuclease mapping (1), in which a DNA probe is hybridized to the RNA of interest, and unhybridized regions are digested with the single-strand specific nuclease S1. For example, in this technique, a given cDNA probe is fully protected from nuclease digestion only by a complementary mRNA, while unspliced or alternatively spliced RNAs derived from the same gene are protected only until the point of sequence divergence. Appropriate DNA clones are necessary to detect all possible products of RNA splicing. In addition, there are a number of potential complications with S1 analysis that can result in an artifactual protection indicative of spliced RNA. These include the formation of trimolecular hybrids in which two different RNA molecules hybridize simultaneously to different regions of the same DNA molecule and fully protect it from nuclease cleavage (2). Full protection of intronless cDNA probes by intron-containing RNA can also occur due to the inability of S1 nuclease to cleave the cDNA probe opposite the unhybridized intron loop (3).

RNA splicing can also be analyzed by primer extension (4) in which a DNA fragment is annealed to RNA sequences downstream of the splice site and extended towards the 5' end of the RNA by reverse transcriptase. The length of the extension

product should be indicative of the presence or absence of the intron. However, the usefulness of this approach is limited by the tendency of reverse transcriptase to terminate prematurely, particularly when the anticipated extension product is greater than several hundred nucleotides in length. Thus primer extension analysis is effectively limited to introns located near the 5' end of the pre-mRNA.

The enzyme RNase H selectively degrades the RNA strand of an RNA:DNA hybrid (5,6). Therefore site-specific cleavage of an RNA molecule can be achieved by hybridization with a complementary oligodeoxynucleotide followed by treatment with RNase H (7). This approach has been successfully applied to RNA sequence, structural, and functional analyses (8-14). Recently the use of oligodeoxynucleotide-directed RNA cleavage coupled with nuclease S1 analysis has been presented (3). In this study, oligodeoxynucleotide-directed RNA cleavage is coupled with primer extension to analyze the products of *in vivo* RNA splicing. The results demonstrate the usefulness of this technique for the analysis of splice site selection.

MATERIALS AND METHODS

Materials

Restriction enzymes, Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, T4 DNA ligase, SP6 polymerase, and nuclease Bal 31 were from New England Biolabs. Nuclease S1 and RNase H were from Boehringer Mannheim. Linkers were from PL Biochemicals. RNasin and RNase-free DNase were from Promega Biotec. Radiolabelled nucleotides were from New England Nuclear. Reverse transcriptase was from Life Sciences, Inc.

Plasmid constructions

In order to generate DNA templates for *in vitro* transcription with SP6 polymerase, the cDNA insert fragments were isolated from the full-length fibroblast Tm 1 and skeletal muscle beta Tm cDNA clones and then ligated to pSP65 DNA. The constructions used for transfection assays contained portions of the cloned rat tropomyosin gene and cDNA in a eukaryotic expression vector, pXKH (D. Hanahan, unpublished work), which contains the SV40 early promoter and origin of replication and a functional poly (A) site (see Figures 5 and 7). The construction designed to analyze the internal alternatively spliced region of the rat tropomyosin 1 gene contains a 1.0 kb Hae II/Cla I fragment which extends from the middle of exon 5 through the middle of exon 9. This fragment was modified with Hind III linkers and cloned into the unique Hind III site located 70 bp downstream of the transcriptional start site of plasmid pXKH. In order to generate the construction designed to analyze the 3' alternatively spliced region, the full-length fibroblast Tm 1 cDNA (15,16) insert was modified with Hind III linkers and cloned into the Hind III site of plasmid pXKH. To replace the cDNA sequences downstream of

amino acid 254 with genomic sequences, this construction was then digested with Cla I and Kpn I and ligated to a Cla I-Kpn I fragment purified from the genomic tropomyosin clone (16) which contains exons 9 through 11 plus flanking introns and about 200 bp downstream of exon 11. The initial deletion of sequences within intron 9 was accomplished by digestion with Eco RI and religation. This removed a 350 bp Eco RI fragment in the 5'-proximal region of the intron. Further deletions were obtained by digestion with Eco RI, followed by digestion with nuclease Bal 31 (0.5 unit/3 micrograms of DNA) for various times. The DNA was then treated with Klenow fragment to generate blunt ends, ligated with Eco RI linkers, recut with Eco RI and religated. The deletion endpoints were mapped by restriction digestion and agarose gel electrophoresis.

Oligodeoxynucleotide hybridization and RNase H digestion

RNA was hybridized to oligodeoxynucleotides complementary to sequences encoding amino acids 223-229 within exon 8 (5' TCCAGAAGTTTGATCTCCTT 3') or amino acids 181-185 within exon 5 (5' CACCTCGGCTCTCTC 3') of the rat tropomyosin (Tm) 1 gene. The oligodeoxynucleotide was present in a 10-100 fold molar excess, and hybridization was performed in 30 microliters of RNase H buffer (50 mM Tris Cl pH 8.3, 70 mM KCl, 25 mM MgCl₂, and 500 micrograms/ml BSA) by incubation at 55-65°C for 3 minutes followed by 30 minutes at 37°C [modified from (7)]. RNase H (1 unit) was then added and reactions were incubated at 37°C for an additional 30 minutes. Reactions were terminated by two extractions with phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation.

Primer extension analysis

Primer extension analysis was carried out essentially as described (4,16). Oligodeoxynucleotides complementary to sequences encoding amino acids 223-229 within exon 8 (5' TCCAGAAGTTTGATCTCTTC), amino acids 261-268 within exon 10 of skeletal muscle beta Tm (5' TGTACTTCATCTTCTGTGCA 3') and amino acids 261-268 within exon 11 of fibroblast Tm 1 (5' CGTTCTCTTCCTTGGCACTA 3') were used as primers (15,16). Primers were end-labelled with (gamma-³²P)ATP using T4 polynucleotide kinase. The primers were annealed to the RNA by incubation for 3 minutes at 55°C, then 30 minutes at 37°C in 30 microliters of primer extension buffer (50 mM Tris Cl pH 8.3, 140 mM KCl, 10 mM MgCl₂). For primer extension, reactions were transferred to 42°C and supplemented with DTT (final concentration of 5 mM) and deoxynucleotide triphosphates (final concentration of 0.5 mM) and 10 U of reverse transcriptase. After 30 minutes, the reactions were terminated by the addition of EDTA to a final concentration of 25 mM, and the reaction products were precipitated with ethanol and analyzed by electrophoresis on an 8% polyacrylamide, 8M urea gel followed by autoradiography. For primer extensions performed on radiolabelled SP6 RNA (Figure 3), the RNA was degraded by treatment with RNase A prior to electrophoresis.

S1 nuclease analysis

S1 nuclease analysis was performed essentially as described (1,16). Full-length skeletal muscle beta Tm and fibroblast Tm 1 cDNA clones (15,16) were cut at the Ava I site corresponding to amino acid 237 in the common exon 9 (see Figure 2) and end-labelled with the Klenow fragment of DNA polymerase I in the presence of (alpha-³²P) dGTP, dCTP, and dTTP. The end-labelled DNAs were then digested at the unique Hind III site in the polylinker downstream of the cDNA insert, and the insert fragments were isolated by agarose gel electrophoresis. The purified end-labelled fragments were then denatured in the presence of 5 to 30 micrograms of total cellular or cytoplasmic RNA at 85°C for 15 minutes in 30 microliters of buffer containing 80% formamide, 0.4 M NaCl, and 10 mM 1,4-piperazinediethanesulfonic acid (pH 6.4). Hybridizations were carried out at 55°C for at least 3 hours. After hybridization, the samples were diluted by the addition of 300 microliters of ice-cold buffer containing 0.28 M NaCl, 0.05 M sodium acetate (pH 4.6), 4.5 mM ZnSO₄, 20 micrograms carrier DNA/ml, and 500 U/ml of S1 nuclease. After incubation at 37°C for 1 hour, the nuclease-resistant fragments were recovered by ethanol precipitation and analyzed by electrophoresis on a 6% acrylamide, 8M urea gel followed by autoradiography.

Cell culture and transfection

COS cells (17) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS. COS cells in monolayer cultures on 100 mm dishes were transfected with 2 micrograms of plasmid DNA in serum-free Hepes-buffered medium plus 250 micrograms/ml DEAE dextran (18). After 1 hr, DNA was removed and replaced with medium plus 0.1 mM chloroquine (19). After 4-5 hours the chloroquine was removed, cells were washed and refed with DMEM plus 5% FBS. Cells were harvested 48 hours later.

RNA extraction

Total cellular RNA was prepared from rat smooth muscle (stomach) and skeletal muscle (thigh) by a modified guanidine thiocyanate procedure (20,21). Total cytoplasmic RNA was isolated from cultured cells following transfection as described (22).

SP6 transcription

The full-length skeletal muscle beta Tm and fibroblast Tm 1 cDNA (15,16) inserts were subcloned into the vector pSP65 and linearized with Hind III. SP6 transcripts (24) were prepared as described by the manufacturer (Promega Biotec) using (alpha-³²P)UTP.

RESULTS

Using a modification of standard techniques we have developed a new method to study pre-mRNA splice site selection. The procedure is diagrammed in Figure 1. First, an oligodeoxynucleotide complementary to exon sequences upstream of the 5' splice site

Figure 1. Diagram of method used to detect RNA splicing. Boxes represent exons, and solid lines represent introns of a hypothetical gene.

of interest is annealed to the RNA. The RNA:DNA hybrid is then digested with RNase H, resulting in RNA molecules that are all cleaved at precisely the same site. A ^{32}P -labelled primer complementary to exon sequences downstream of the 3' splice site is then annealed to the RNA and extended towards the 5' end by reverse transcriptase. The primer extension products are separated by polyacrylamide gel electrophoresis and visualized by autoradiography. In theory, the length of the resulting fragments will be indicative of correctly spliced, incorrectly spliced, or unspliced RNA molecules.

To establish the applicability of this method, we have used this technique to characterize RNA molecules expressed from a rat tropomyosin gene. Previous work from our laboratory has indicated that a single gene encodes two tropomyosin isoforms via tissue-specific RNA processing (16). The skeletal muscle beta Tm isoform is expressed exclusively in skeletal muscle, and the fibroblast Tm 1 isoform is expressed in fibroblasts as well as smooth muscle. The complete structure of this gene has been determined and is presented in schematic form in Figure 2. As shown in Figure 2, the two Tm mRNAs are the result of alternative internal exon selection as well as alternative 3' exon selection and poly (A) site utilization.

Conditions for oligodeoxynucleotide hybridization, RNase H cleavage and primer extension analysis were first determined using full-length RNAs encoding skeletal

Figure 2. Schematic diagram of the tropomyosin gene and model for the generation of rat embryonic fibroblast Tm 1 and skeletal muscle beta Tm mRNAs by alternative RNA processing. Open boxes represent common exons, hatched boxes represent fibroblast or smooth-muscle exons, closed boxes represent skeletal muscle exons, and horizontal lines represent introns; they are not drawn to scale. The amino acids encoded by each exon are indicated. The cap site and polyadenylation signal AATAAA are also indicated.

muscle beta Tm and fibroblast Tm 1 synthesized *in vitro* by the SP6 transcription system. These RNAs were first annealed to an oligodeoxynucleotide complementary to codons 223-229 within the common exon 8. The RNA:DNA hybrids were then digested with RNase H. This treatment yields 2 RNA fragments which represent the sequences 5' and 3' of the site of RNase H cleavage. The cleavage is dependent upon prior oligodeoxynucleotide hybridization, as incubation of the RNA with RNase H in the absence of a complementary oligodeoxynucleotide has no effect on either SP6 transcript (data not shown). These RNAs were then subjected to primer extension analysis using oligodeoxynucleotide primers complementary to codons 261-268 within the alternative 3' exons 10 (skeletal muscle beta Tm) and 11 (fibroblast Tm 1). Both of these primers are specific for either exon 10 or 11 (presented in Figure 4). When uncleaved fibroblast Tm 1 and skeletal muscle beta Tm RNAs are used as substrates, the primer extension reaction proceeds to the 5' end of the transcript. The resulting extension products are 870 nt and 820 nt, respectively (Fig. 3, lanes 2 and 4, indicated by arrows). The differences in the length of the primer extension products are due to the amount of 5' untranslated sequence present in the original cDNAs. By contrast, when RNAs subjected to prior oligodeoxynucleotide hybridization and RNase H cleavage are used as substrates for primer extension, the extension reaction is terminated at the site of nuclease cleavage. In this instance, the predicted extension product of 116 nt originates at codon 268 and terminates at codon 229. Primer extension of precleaved fibroblast Tm 1 and skeletal muscle beta Tm transcripts, shown in lanes 1 and 3, yields a major product of 116 nt. The few bands slightly larger than 116 nt probably represent some heterogeneity in the region of nuclease cleavage, and the smaller bands represent premature termination by reverse transcriptase. The full-length extension product is no longer detected following the oligonucleotide/RNase H treatment, indicating efficient cleavage of all of the RNA.

Figure 3. Primer extension of SP6 transcripts with and without prior oligodeoxynucleotide hybridization and RNase H digestion. Fibroblast Tm 1 and skeletal muscle beta Tm cDNAs were subcloned into pSP65 and transcribed *in vitro*. Seven ng of each RNA was hybridized with a 10 fold molar excess of the oligodeoxynucleotide complementary to exon 8, and then digested with one unit of RNase H. After digestion, the purified RNA subjected to primer extension using end-labelled primers complementary to exon 10 of skeletal muscle beta Tm and exon 11 of fibroblast Tm 1. The reaction products were analyzed by electrophoresis on an 8% acrylamide, 8M urea gel, followed by autoradiography for 18 hours. Lane 1, fibroblast Tm 1 RNA with oligodeoxynucleotide-RNase H cleavage; lane 2, fibroblast Tm 1 RNA without prior cleavage; lane 3, skeletal muscle beta Tm RNA with oligodeoxynucleotide-RNase H cleavage; lane 4, skeletal muscle beta Tm RNA without prior cleavage. The extension products derived from uncleaved RNA (870 nt and 820 nt) and precleaved RNA (116 nt) are indicated.

Figure 4. Panels A and B: Primer extension analysis of rat smooth and skeletal muscle RNA. Ten micrograms of RNA isolated from rat smooth and skeletal muscle was hybridized with the oligodeoxynucleotide complementary to exon 8, digested with RNase H, and subjected to primer extensions using primers complementary to exons 10 and 11. The reaction products were analyzed by electrophoresis on an 8% acrylamide, 8M urea gel followed by autoradiography for 18 hours. Panel A refers to smooth muscle RNA, panel B refers to skeletal muscle RNA. Lane 1, fibroblast Tm 1 (exon 11) primer, no RNase H cleavage; lane 2, skeletal muscle beta Tm (exon 10) primer, no RNase H cleavage; lane 3, prior cleavage at exon 8, fibroblast Tm 1 primer; lane 4, prior cleavage at exon 8, skeletal muscle beta Tm primer. The 116 nt extension product is indicated. Panels C and D: S1 nuclease analysis of rat smooth and skeletal muscle RNA. Five micrograms of RNA isolated from rat smooth and skeletal muscle was hybridized with S1 probes derived from the fibroblast Tm 1 and skeletal muscle beta Tm cDNA clones. The probes contain either exons 9 and 11, or 9 and 10, plus a small amount of vector DNA. The full-length fibroblast Tm 1 probe is 249 nt, the nuclease resistant fragment is 215 nt. The full-length skeletal muscle beta Tm probe is 415 nt, the nuclease resistant fragment is 381 nt. The reactions were then analyzed by electrophoresis on a 6% acrylamide 8M urea gel, followed by autoradiography for 18 hours. Panel C, fibroblast Tm 1 probe; panel D, skeletal muscle beta Tm probe. Lane 1, unreacted probe; lane 2, no added RNA; lane 3, smooth muscle RNA; lane 4, skeletal muscle RNA.

As previously mentioned, one problem associated with primer extension analysis is the inability to obtain full-length products, especially when the expected product is greater than a few hundred nucleotides in length. Thus, although we were able to detect primer extension products corresponding to the full-length RNA, the ratio of full-length primer extension product to oligo-cleavage-terminated product is approximately 1:10, as indicated by the relative intensity of the products in the autoradiograph (Figure 3). Although we have not encountered any problem, it is possible that the efficiency of RNase H cleavage may vary depending on the oligodeoxynucleotide itself. For example, the oligodeoxynucleotide may be complementary to a region of the RNA that is inaccessible due to secondary structure. In this instance, it may be necessary to try oligodeoxynucleotides complementary to a different region. .

This approach was then used to characterize tropomyosin mRNAs expressed in rat skeletal muscle and smooth muscle (Figure 4). RNA isolated from smooth muscle and skeletal muscle was hybridized to the oligodeoxynucleotide complementary to exon 8, digested with RNase H, and subjected to primer extension using primers complementary to exons 10 and 11 as described in Figure 3. Again, the predicted product of 116 nt is observed in RNAs subjected to oligodeoxynucleotide-directed hydrolysis prior to primer extension with the appropriate exon primer (Fig. 4A, lane 3, and 4B, lane 4). This product is not observed in the primer extension of RNA molecules which have not been previously cleaved (4A and 4B, lanes 1 and 2). The specificity of the primers is illustrated by the fact that no extension product is observed when the skeletal muscle beta Tm exon primer is used in combination with smooth muscle RNA, and vice versa (4A, lane 4 and 4B, lane 3, respectively). For comparative purposes RNA isolated from skeletal and smooth muscle was also subjected to S1 nuclease analysis using probes derived from the 3' region of the skeletal muscle beta Tm and fibroblast Tm 1 cDNA clones. These probes contain sequences corresponding to either exons 9 and 10, or 9 and 11, respectively, plus a small amount of vector DNA. As seen in Figure 4C and 4D, the S1 probe derived from the fibroblast Tm 1 cDNA is protected only by RNA from rat smooth muscle (4C, lane 3) and the probe derived from the skeletal muscle beta Tm cDNA is protected by RNA from skeletal muscle (4D, lane 4). A fragment that is protected only until the end of the sequences common to both smooth muscle and skeletal muscle tropomyosin mRNAs in exon 9 is never observed. This fragment is 61 nt in length with an A:T content of 52%, and we suspect that this fragment is unstable under these reaction conditions.

One approach to study alternative RNA processing is to introduce various regions of the tropomyosin gene into cultured cells and analyze the RNA splice products generated following transient expression. We designed experiments to determine whether oligodeoxynucleotide-directed RNA hydrolysis coupled with primer extension

Figure 5. Diagram of tropomyosin 3' minigene constructions. The initial construction was created by the ligation of tropomyosin cDNA and genomic DNA into an expression vector, as described in methods. Additional constructions were generated by limited nuclease digestion, as described.

would be applicable to the analysis of RNAs generated by this method. A number of minigene constructions designed to analyze 3' end processing were prepared and transfected into cultured cells. These constructions are diagrammed in Figure 5. The initial minigene construction contains a portion of the fibroblast Tm 1 cDNA extending from the 5' untranslated sequence to a restriction site within exon 9 ligated to a genomic tropomyosin DNA fragment containing exons 9 through 11 plus flanking introns and sequences downstream of exon 11. Two additional constructions contain either a small or a large deletion in intron 9, the intron preceding the alternatively spliced exons. RNA isolated after transient expression of these minigenes in cultured COS cells was characterized by oligodeoxynucleotide-directed RNase H cleavage followed by primer extension and by S1 nuclease analysis.

Analysis of these RNAs by primer extension is shown in Figure 6A and 6B. The RNAs were hybridized to the oligodeoxynucleotide complementary to exon 8, digested with RNase H, and subjected to primer extension using primers complementary to exons 10 (skeletal muscle beta Tm) and 11 (fibroblast Tm 1) as described above. The 116 nt extension product is observed with both the fibroblast-specific and skeletal-muscle specific primers (panels A and B, respectively) in RNAs isolated from COS cells transfected with the parent minigene construction (lane 4), and the two deletion derivatives (lanes 6 and 8), but not in mock-transfected cells (lane 2). The same extension product is also observed in RNA isolated from rat smooth and skeletal muscle (lane 10). This product is not observed when primer extension reactions are performed on the same RNAs without prior oligodeoxynucleotide hybridization and RNase H cleavage (lanes 1, 3, 5, 7, and 9).

In order to determine whether these results are qualitatively similar to those obtained by S1 nuclease analysis, RNA isolated from cells after transient expression of the same minigene constructions was subjected to S1 nuclease analysis. The S1 probes are identical to those used to analyze the endogenous rat mRNAs (Fig. 4C and 4D).

Panel C and D: S1 nuclease analysis of RNA generated by transient expression of tropomyosin 3' minigenes in cultured cells. RNA isolated from transfected cells (ten micrograms) and rat smooth and skeletal muscle (ten micrograms) was subjected to S1 analysis as described in Figure 4. Panel C, fibroblast Tm 1 probe, panel D, skeletal muscle beta Tm probe. Lane 1, unhybridized S1 probe; lane 2, S1 hybridization with no added RNA; lane 3, RNA from mock-transfected cells; lane 4, transfection with the initial minigene construction; lane 5, transfection with small deletion derivative; lane 6, transfection with the large deletion derivative; lane 7, smooth muscle RNA; lane 8, skeletal muscle RNA.

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Figure 7. Primer extension analysis of RNA generated by transient expression of the tropomyosin internal minigene in cultured cells. RNA isolated from transfected cells (five micrograms) and rat smooth and skeletal muscle (five micrograms) was hybridized with the oligodeoxynucleotide complementary to exon 5, digested with RNase H, and subjected to primer extension using a primer complementary to exon 8. The reaction products were analyzed on an 8% acrylamide, 8M urea gel followed by autoradiography for two days. Lanes 1, 3, 5, and 7, reactions without prior oligodeoxynucleotide-directed cleavage; lanes 2, 4, 6, and 8, reactions following oligodeoxynucleotide-directed cleavage. Lanes 1 and 2, RNA isolated from mock-transfected cells; lanes 3 and 4, RNA isolated from cells transfected with the internal minigene; lanes 5 and 6, smooth muscle RNA; lanes 7 and 8, skeletal muscle RNA. The primer extension products corresponding to the splicing of exons 5+8 ("A", 55 nt), and exons 5+6+8 and 5+7+8 ("B" and "C", 131 nt) are indicated.

isolated from mock-transfected cells (lane 3). The results obtained by both methods are qualitatively the same, demonstrating that RNase H cleavage followed by primer extension can be used to analyze RNA splice products generated by transient expression.

The generation of two tropomyosin isoforms from the Tm 1 gene requires alternative internal exon selection as well as 3' end exon selection (illustrated in Figure 2). A minigene containing genomic sequences extending from exon 5 through exon 9, including the internal alternatively spliced exons 6 (fibroblast-specific) and 7 (skeletal muscle-specific), was also introduced into COS cells. RNA isolated after transient expression was analyzed by oligodeoxynucleotide directed-RNase H cleavage followed by primer extension, as shown in Figure 7. RNA was hybridized to an oligodeoxynucleotide complementary to sequences within exon 5, digested with RNase H, and then subjected to primer extension using an oligodeoxynucleotide complementary to sequences within exon 8 as a primer. Rat smooth muscle and skeletal muscle RNAs were included as controls. The extension product "B or C" observed in these reactions (Figure 7, lanes 6 and 8, respectively) is 131 nt in length and represents splicing of exons 5+6+8 in smooth muscle RNA, and 5+7+8 in skeletal muscle RNA. This size product is also observed in RNA isolated from cells transfected with the minigene (lane 4), but not in RNA isolated from mock-transfected cells (lane 2). Therefore, transient expression of this minigene results in splicing of 5+6+8, and/or 5+7+8, as these extension products are indistinguishable in this experiment. In order to determine the relative utilization of exon 6 and exon 7 in COS cells, we performed two additional experiments (data not shown). First, when RNA isolated from cells transfected with this minigene is cleaved at exon 5 and subjected to primer extension using primers complementary to sequences within the alternative exons 6 and 7, roughly equal amounts of the 5+6 and 5+7 splice products were detected. Second, cleavage at exon 6 or exon 7 followed by primer extension using an exon 8 primer showed roughly equal amounts of the 6+8 and 7+8 splice products. In addition, cleavage at exon 6 followed by primer extension using the exon 7 primer gave no detectable product corresponding to splicing of the two alternative exons 6 and 7 to one another. Also of note in Figure 8 is the extension product "A", which is 55 nt in length and is observed only in RNA isolated from cells transfected with the minigene. This product corresponds to splicing of exon 5 directly to exon 8. This product is not observed in smooth muscle and skeletal muscle RNA (lanes 6 and 8, respectively), suggesting that this "exon skipping" does not naturally occur in these tissues.

DISCUSSION

The experiments presented above demonstrate a new method to analyze RNA splicing patterns. In this study we show that site-specific RNA cleavage can be

combined with primer extension to identify RNA splice products. We have used this technique to analyze rat tropomyosin mRNA expressed in different tissues and RNAs generated *in vivo* by transient expression of several rat tropomyosin minigene constructions in cultured cells. Preliminary results suggest that this approach will also be applicable to the analysis of tropomyosin pre-mRNA splicing *in vitro* (unpubl.)

The results of both S1 nuclease and primer extension analyses demonstrate that the RNAs transcribed from the 3' minigene constructions are processed in COS cells. This suggests that the genomic sequences present are sufficient for the utilization of the common 5' splice site and both of the alternative 3' splice sites. As shown, splicing to both of the alternative exons is observed, with an apparent preference for the more distal fibroblast-specific exon 11. Further, deletions within the intron preceding the alternatively spliced exons do not appear to affect the relative utilization of the downstream splice acceptor sites. With regard to the internal minigene, primer extension products corresponding to exons 5+6+8, 5+7+8, and 5+8 were detected following transfection in COS cells. These results demonstrate that these minigenes are not processed in a cell-type specific fashion in COS cells. This may be due to the absence of additional 5' and 3' sequences which may be required for appropriate splicing. Alternatively, the lack of any cell-type specificity in COS cells could indicate that the utilization of tissue-specific exons does not require tissue-specific processing factors. It is also possible that since these minigenes contain the SV40 origin of replication, transfection into COS cells results in a very high copy number of plasmid DNA per cell, leading to a large number of tropomyosin pre-mRNA transcripts. If splicing is regulated by a limited number of factors, the high level expression may lead to permissive splice site selection by competing out such putative trans-acting regulators. Experiments are currently underway to assay the splice products generated from these and other minigene constructions *in vivo* following expression in rodent fibroblasts and myogenic cell lines.

The method presented in this manuscript is similar to one used to analyze the effect of viral deletions and mutations on the expression of the SV40 early region, which involves multiple transcriptional start sites and alternative splicing pathways (23). Viral RNAs were subjected to primer extension and the resulting cDNAs were hybridized to single-stranded DNA derived from a restriction fragment spanning the 5' portion of the SV40 late region. Digestion with appropriate restriction enzymes produced cDNAs with flush 3' ends regardless of the transcription start site, and the relative abundance of the various cDNAs could be correlated with the relative utilization of the many splice pathways. The major difference between these two approaches is that in our approach RNA is cleaved prior to primer extension, as opposed to cleavage of the cDNA generated by primer extension. Our method, by using site-directed cleavage of the

RNA, obviates the need for producing specific S1 nuclease probes or single-stranded DNAs.

This technique simplifies the analysis of RNA processing of complex as well as simple transcription units. For example, as shown, experiments can be designed either to focus on a single splicing event or to detect the products of multiple splicing events simultaneously. Further, as authentic splicing results in an extension product of a known length, the appearance of additional extension products could be the result of additional splicing events. These splice products could arise by the use of previously undetected splice sites, the activation of cryptic splice sites or by novel pairing of splice sites, i.e. exon skipping, as seen in Figure 7. This technique may be sensitive enough to detect the products of rare splicing events *in vivo*. The identity of all extension products can be directly determined by DNA sequencing. This is particularly advantageous in characterizing splice products generated by transient expression of minigene constructions, as activation of novel splice patterns may result as a consequence of loss of exon and intron sequences and/or expression in heterologous cell lines.

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